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ORIGINAL ARTICLE

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Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*

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Abstract Sex-determining cascades are supposed to have evolved in a retrograde manner from bottom to top. Wilkins' 1995 hypothesis finds support from our comparative studies in *Drosophila melanogaster* and *Musca domestica*, two dipteran species that separated some 120 million years ago. The sex-determining cascades in these flies differ at the level of the primary sex-determining signal and their targets, *Sxl* in *Drosophila* and *F* in *Musca*. Here we present evidence that they converge at the level of the terminal regulator, *doublesex* (*dsx*), which conveys the selected sexual fate to the differentiation genes. The *dsx* homologue in *Musca*, *Md-dsx*, encodes male-specific ($MdDSX^M$) and female-specific ($MdDSX^F$) protein variants which correspond in structure to those in *Drosophila*. Sex-specific regulation of *Md-dsx* is controlled by the switch gene *F* via a splicing mechanism that is similar but in some relevant aspects different from that in *Drosophila.* $MdDSX^F$ expression can activate the vitellogenin genes in *Drosophila* and *Musca* males, and $MdDSX^M$ expression in *Drosophila* females can cause male-like pigmentation of posterior tergites, suggesting that these *Musca dsx* variants are conserved not only in structure but also in function. Furthermore, downregulation of *Md-dsx* activity in *Musca* by injecting dsRNA into embryos leads to intersexual differentiation of the gonads. These results strongly support a role of *Md-dsx* as the final regulatory gene in the sex-determining hierarchy of the housefly.

Keywords *Musca domestica* · Sex determination · *doublesex* · Alternative splicing

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Introduction

Insects employ a variety of seemingly different strategies to determine sex (Nothiger and Steinmann-Zwicky 1985). This variability becomes evident even in one single species, the housefly *Musca domestica*, where different modes of sex determination have been found in naturally occurring populations (Milani 1967). These observations suggested the presence of a short genetic cascade for the control of sexual development in *Musca*: a dominant male-determining factor, *M*, represses the key gene for sex determination, *F*, which leads to male development. Absence of *M* and presence of maternal *F* product in the zygote are the prerequisites for *F* activity, which results in female development (for review see Dubendorfer et al. 2002).

In most wild strains, *M* is located on the Y chromosome, but strains in which both sexes are XX and males carry an autosomal *M* in heterozygous condition also exist (Rubini et al. 1972). Other wild populations are known where both sexes are homozygous for *M*, and females are heterozygous for the dominant gain-of-function allele F^D which overrules the male-determining function of *M* (Milani 1967). Yet another type of sex-determining system operates without *M*. In this strain, which arose in our laboratory, males are homozygous for a putative recessive loss-of-function mutation of *F*, F^{man}, whereas females are heterozygous for the mutation (Schmidt et al. 1997). Finally, Vanossi Este and Rovati (1982) described a system of maternally controlled sex determination, where *Ag*/+ females, due to a failure to activate *F* in the germline (Hilfiker-Kleiner et al. 1993), are arrhenogenic and produce sons, whereas +/+ females are thelygenic and, with males of this strain, produce exclusively daughters. We have proposed that all these different modes of sex determination in *M. domestica* are based on single mutations in an otherwise invariant set of genes, rather than on major alterations in the genetic architecture

of the pathway that controls sexual development (Dubendorfer et al. 2002).

To identify the regulatory components involved in *Musca* sex determination, we initiated a comparative analysis with the well-characterised cascade of *Drosophila melanogaster* as a reference. Our objective was to isolate genes in *Musca* based on homology to the sex-determining genes in *Drosophila* and to test them for a possible role in sexual differentiation. This type of analysis is expected to unveil the extent of correspondence between the two sex-determining pathways. Dissimilarities are found in the primary signal that determines the sexual fate of the embryo. *Drosophila* does not employ dominant male or female determiners, but rather the number of X-chromosomes relative to sets of autosomes, the X:A ratio, as a primary signal for sex determination (Cline 1993). Genetic studies in *Musca* revealed a binary switch gene, *F*, which is controlled by the primary signal *M*. The functional correspondence of Sex-lethal (*Sxl*) and *F* does not, however, extend to the molecular level. In a previous study, we have demonstrated that the *Musca* homologue of *Sxl* is equally expressed in both sexes which makes it an unlikely candidate for *F* (Meise et al. 1998). The possibility remains that *F* corresponds to one of the more downstream genetic switches in the pathway of *Drosophila*.

In *Drosophila*, *Sxl* transduces the selected fate—female when on and male when off—through the switch gene *transformer* (*tra*) to the last gene in the cascade, *double sex* (*dsx*; reviewed in Baker et al. 1989). *dsx* encodes two sex-specific variants of a transcription factor that share an atypical zinc finger domain, but differ in their carboxy-terminal parts (Baker et al. 1989; Erdman and Burtis 1993). The male and female proteins control transcription of the terminal differentiation genes with opposite activities. The female-specific DSX protein, DSX^F, directs female development by promoting transcription of female-specific differentiation genes and repressing male-specific differentiation genes, while the male-specific DSX protein, DSX^M, acts in the opposite direction (reviewed in Baker et al. 1989; Slee and Bownes 1990). Sex-specific expression of *dsx* is achieved at the level of differential splicing and depends on the activity of *tra* and *transformer2* (*tra2*). In females, active TRA products, in combination with TRA2 proteins, bind to splice enhancer sites in the *dsx* pre-mRNA to promote the use of a weak female-specific 3′ acceptor site (Hedley and Maniatis 1991; Hertel et al. 1996; Ryner and Baker 1991; Tian and Maniatis 1993). This splice pattern leads to the inclusion of an exon that encodes the female-specific carboxy end (Fig. 1a). In males, where no active TRA is present, this site is not recognized, and two downstream exons are included that encode the male-specific carboxy end (Fig. 1a).

In this study, we have identified the *dsx* homologue in *Musca*, *Md-dsx.* Our results confirm a role of *Md-dsx* in sexual differentiation of the housefly. Sex-specific regulation of *Md-dsx* is achieved at the post-transcriptional level, but shows some interesting deviations from the mechanism that operates in *D. melanogaster.*

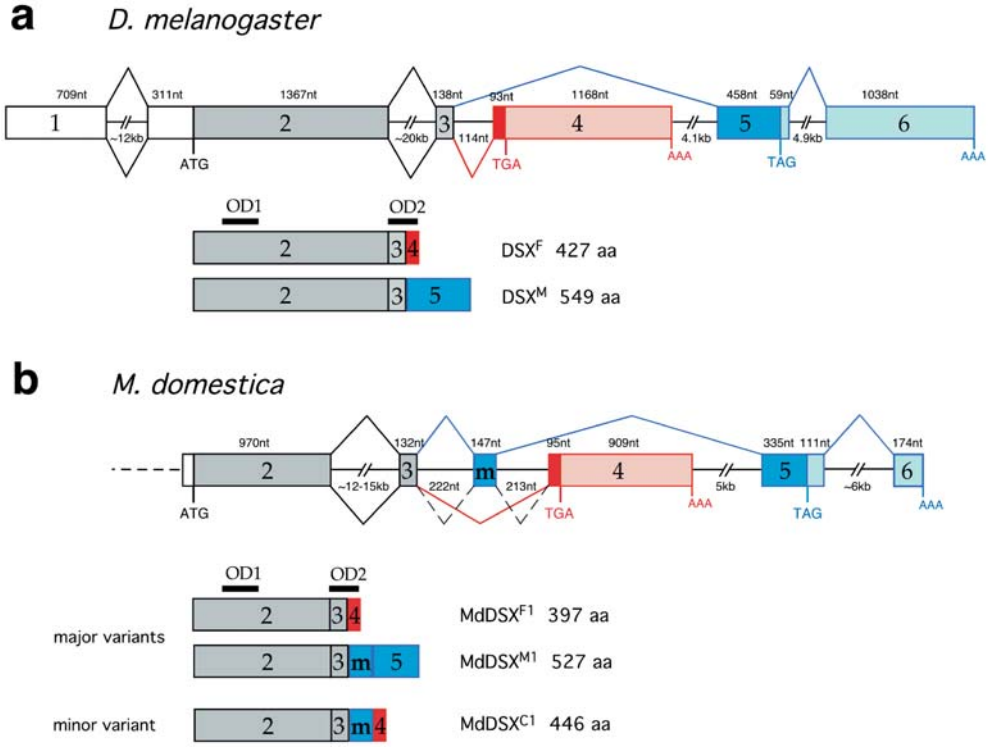

Fig. 1 Schematic drawing of the genomic organization and the structure of splice variants of *dsx* in *Drosophila* (**a**) and *Musca* (**b**). Male-specific exons are marked in *blue* and the female-specific exon in *red*. Note that exon m in the *Musca* gene has no correspondence in the *Drosophila* gene. Exon and intron sizes are indicated in nucleotides (nt), translational start and stop sites as well as the poly(A) addition sites are *marked*

Materials and methods

PCR with degenerated primers

The 5′ primers correspond to sequences located in exon 3, and 3′ primers in the female-specific exon 4 of the *dsx* gene of *D. melanogaster.* One pair of degenerated primers, DSXC and DSXF2, was kindly provided by Dr. Guiseppe Saccone (University of Naples, Italy) and another pair, FHC3 and FHC4, we obtained from Dr. Antonio Pannuti (Emory University, Atlanta, United States).

DSXC 5′	GAR AAR TTY MGY TAY CCI TGG
DSXF2 3′	DAT RTT IAR RTT RTG YTG IC
FHC2 5′	CTI (CT)TI GA(GA) AA(AG) TT(TC) (CA)GI TA(TC) CCI TGG
FHC4 3′	T(GT)(TC) TGI C(GT)I GA(AG) TA(TC) TC(AG) TTI ACI AC

Musca templates were prepared from male and female cDNA of our wild-type (XX; XY) strain. A first PCR round was performed with DSXC and DSXF2 primers followed by a second amplification with FHC2 and FHC4. We used standard concentrations of Mg^{++} and nucleotides (Expand Long Template PCR System, Boehringer Mannheim). In a total volume of 50 µl, 50 ng DNA template was amplified with 20 µM of each primer. The following conditions were used: denaturation at 95°C for 5 min with subsequent addition of Taq polymerase, then 35 cycles (denaturation 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min), and lastly extension at 72°C for 7 min. Subcloning and sequencing of the candidate fragments were carried out by standard procedures.

Rearing of *Musca* strains

Strains were reared as described previously (Schmidt et al. 1997). Since small populations of larvae are difficult to raise on standard medium, larvae obtained from injected embryos were raised on porcine faeces. To kill *Musca* eggs and larvae of natural populations, dung was frozen at –20°C for several days and thawed before supplementing to standard medium.

Strains of *M. domestica*

The strains were: (1) wild-type strain, females *XX; +/+*, males *XY; +/+*; (2) NoM strain, females *XX;* F^{man}/F^{+}, males *XX;* F^{man}/F^{man}; (3) F^{D} strain, females M^{III}/M^{III} ; F^{D}/F^{+}, males M^{III}/M^{III}; F^{+}/F^{+}; (4) *white* strain, females *XX; w/w*, males *XY; w/w*; (5) autosomal *M* strain, females *XX; pw bwb / pw bwb*, males *XX;* M^{III} pw^{+} bwb^{+}*/pw bwb*; (6) NoM strain, females *XX; +/+* (thelygenic) or *XX; Ag/+* (arrhenogenic), males *XX; +/+* or *XX; Ag/+* (Vanossi Este and Rovati 1982).

Crosses to obtain unisexual progenies were: female only (a) virgin females of strain 1 × males of strain 6, or (b) virgin females of strain 1 × males of strain 2; male only (c) virgin females of strain 1 × males of strain 3.

Northern blot analysis

Total RNA of about 200 mg flies [14 adult males (cross c) or 12 adult females (cross b)] was extracted with the AGPC-technique (Chomczynski and Sacchi 1987). Poly(A)$^{+}$ RNA was isolated using the Oligotex mRNA Maxi Kit of Qiagen. Poly(A)$^{+}$ RNA (10 µg per lane) was electrophoresed on a 1% agarose gel using the glyoxal technique. RNA was transferred by blotting with 10× SSPE to Hybond-N^{+} nylon membranes (Amersham). Filters were preincubated for 2 h at 42°C in hybridization buffer (50% formamide, 4× SSPE buffer, 5× Denhardt's solution, 1% SDS, 10 mM Tris-HCl pH 7.5, 0.1 mg/ml salmon sperm). Filters were then subsequently incubated overnight at 42°C in hybridization buffer containing 10^{6} cpm/ml radiolabeled RNA probes. RNA probes were generated by in vitro transcription of a cDNA template of the common part of *dsx* (*dsx*C) in the presence of [α-^{32}P]-CTP and T7 RNA polymerase. Filters were washed twice for 15 min in 2× SSPE at room temperature, then twice for 45 min in 2× SSPE/2% SDS at 65°C and finally two times in 0.1× SSPE for 15 min at room temperature. Filters were exposed overnight or longer on Fuji RX films.

RT-PCR analysis

The total RNA of three adult flies (around 50 mg) was extracted according to the RNeasy Mini protocol of Qiagen. RT-PCR reactions were performed using the Titan One Tube RT-PCR Kit (Roche) and *Md-dsx*-specific primers following the manufacturer's instructions.

Transgenic constructs and germline transformation

Drosophila

The *Drosophila* transformation vectors pUAST-*Md-dsx*F1 and pUAST-*Md-dsx*M1 were created by introducing a 1.4-kb *Eco*RI/*Xba*I cDNA fragment of *Md-dsx*F1 and a 1.8-kb cDNA fragment of *Md-dsx*M1 containing the whole open reading frames into the *Eco*RI/*Xba*I sites of the pUAST vector (Brand and Perrimon 1993).

The pHermes{Act5C-*Md-dsx*M1} construct was made by introducing the 1.8-kb *Md-dsx*M1 *Eco*RI/*Xba*I cDNA fragment into the *Bam*HI/*Xba*I sites of the pHAct5cEGFP construct (Pinkerton et al. 2000). The pHermes{Act5C-*Md-dsx*M1} construct was co-injected with the helper plasmid pKHSHH (500 ng/µl each) which expresses the Hermes transposase under the control of the *Drosophila* hsp70 promoter (Sarkar et al. 1997). As a host for germline integration *Drosophila* embryos of strain w^{1118} were used.

Musca

The pBac{3xP3-eGFP;hsp82-*Md-dsx*F1} transgene was constructed as follows: an hsp82 promoter/actin5C poly(A) signal fragment was isolated from pKhsp82 (Coates et al. 1996) and a 1.4-kb *Md-dsx*F1 *Eco*RI/*Xba*I cDNA fragment was placed between the hsp82 promoter and the actin5C poly(A) signal. This hsp82-*Md-dsx*F1-actin5C fragment was inserted into the FseI/AscI sites of the pBac{3xP3-eGFP}afm vector (Horn and Wimmer 2000). Twenty micrograms of pBac{3xP3-eGFP; hsp82-*Md-dsx*F1} vector was coprecipitated with 4 µg phsp-pBac helper plasmid containing the pBac-transposase under the control of the hsp70 promoter of *D. melanogaster* (Handler and Harrell 1999) and taken up in a volume of 30 µl injection buffer. Preblastoderm embryos of the *M. domestica* strain 4 were injected as described previously (Hediger et al. 2001).

Injection of dsRNA

cDNA fragments of the common (*dsx*C), female-specific (*dsx*F) and male-specific parts (*dsx*M) of the *Musca dsx* gene were produced by PCR flanked by T7 promoter sequences at their 3′ and 5′ ends. The *dsx*C fragment is part of exon 2 containing the entire OD1 and has a length of 480 bp. The *dsx*F fragment contains coding and noncoding parts of exon 4 and is 640 bp long. The *dsx*M fragment spans exons "m" and 5 and has a length of 560 bp. To produce dsRNA, the three cDNA fragments were transcribed in vitro using T7 RNA polymerase. The dsRNA was ethanol-precipitated and resuspended in injection buffer (final concentration 1 µg/µl).

Embryos were collected 0–1 h after egg laying (preblastoderm stage), dechorionated and injected as described by Hediger et al. (2001). Injected embryos were allowed to develop at room temperature.

Immunoblot analysis

Drosophila

Males carrying the pUAST-*Md-dsx*F1 construct together with the hsp70-GAL4 driver were treated with a twice-repeated heatshock pulse of 1 h at 37°C followed by 3 h at 25°C. Ten males and, for control, five females were then homogenized in 100 µl 2× SDS loading buffer. Samples were boiled for 5 min and insoluble material was removed by centrifugation. Of the supernatant 5 µl was loaded per lane and separated on 12% SDS-PAGE. After electrophoresis, protein was electrotransferred to a nitrocellulose membrane in Tris-glycin-methanol. Membranes were blocked in 4% low-fat dry milk powder in TBS/0.05% Tween-20 (TBST). We used a polyclonal anti-yolk protein antibody from *D. melanogaster* (gift from M. Bownes, University of Edinburgh) at a dilution of 1:5,000 in TBS/0.05% Tween-20/1 mg/ml BSA. For subsequent detection of the antigen-antibody complex, we used the alkaline phosphatase-conjugated anti-rabbit antibody (Promega) at a dilution of 1:7,500.

Musca

Animals carrying the pBac{hsp82-*Md-dsx*F1} construct were kept at 25°C and treated every 5 h with a 1 h heatshock pulse of 42°C from the early embryo stage until 5 days after eclosion. On the sixth day of adult life, 1 µl haemolymph was drawn from a single male with a fine glass needle. The haemolymph was mixed with 12 µl 2× SDS loading buffer and the samples were separated on 12% SDS-PAGE followed by transfer to a nitrocellulose membrane as described earlier. We used a polyclonal anti-yolk protein-antibody from *M. domestica* (kindly provided by Dr. T. Adams, Fargo, N.D.) at a dilution of 1:20,000 in TBS/0.05% Tween-20+1 mg/ml BSA. Detection of the antigen-antibody complex on the blot was done as described earlier.

Results

Isolation of the *dsx* homologue in *M. domestica*

Using a set of degenerated *dsx* primers, we amplified an 84 bp fragment from cDNAs prepared from total RNA of female *Musca* larvae (see Materials and methods). The sequence of this fragment, located between the DM domain (exon 3) and the female-specific domain (exon 4), displays a high degree of sequence similarity at the nucleotide level (68%) and at the amino acid level (82%) when compared to *dsx* sequences of *D. melanogaster.* With this fragment as a probe, two lambda clones (GEM11.14 and GEM11.18) were isolated from a genomic *Musca* DNA library (Tortiglione and Bownes 1997). An alignment of the phage insert sequences with the partial female cDNA sequence exposed an intron at exactly the same position as in *Drosophila* (Fig. 1a, b). This intron is 582 bp long and larger than the corresponding 114 bp intron in *Drosophila* between exons 3 and 4 (Baker et al. 1989). Interestingly, this intron in the *Musca* sequence harbours an additional exon that is preferentially included in transcripts isolated from males (see later). This additional exon has a length of 147 bp and was termed "m" for *Musca*- and male-specific.

To retrieve full-length cDNA sequences, we extended the cDNA fragment on both sides by 5′ and 3′ RACEs. Templates were synthesized from total RNA prepared from male and female third instar larvae. 5′ RACE on female templates led to the isolation of sequences corresponding to exon 2 of *Drosophila dsx;* and 3′ RACE on male cDNA templates revealed two exonic sequences downstream of exon 3 that are not co-linear with the previously isolated exon 4 sequences from female templates (Fig. 1b). We therefore suspected that these sequences are male-specific and termed them exon 5 and 6. Exon 2, 5 and 6 sequences are not contained within the genomic DNA inserts of GEM11.14 and GEM11.18 indicating that the intron between exons 2 and 3 must be larger than 14 kb, and the intron between exons 3 and 5 larger than 4 kb. In *Drosophila*, the intron between exons 2 and 3 has a size of about 23 kb, and the intron between exons 3 and 5 is 4.9 kb (Baker et al. 1989). Given the considerable similarity in structure and organization, we referred to this gene as *Md-dsx*.

Md-dsx encodes protein variants with structural similarity to DSX^M and DSX^F in *Drosophila*

We isolated a cDNA from male flies composed of exons 2-3-m-5-6 and termed this clone *Md-dsx*M1 (Fig. 1b). The major female variant composed of exons 2-3-4 was termed *Md-dsx*F1 (Fig. 1b). Both cDNAs represent two major splice variants of *Md-dsx* (Genbank AY461853 AY461854) and display a high degree of identity at the amino acid level to the corresponding variants in *Drosophila*. *Drosophila* DSX protein essentially consists of two domains, OD1 and OD2, which serve as interfaces for protein and DNA interactions (An et al. 1996; Cho and Wensink 1997). OD1 is composed of an atypical zinc-finger domain (DM) which directly binds to target sequences on the DNA. OD2 is an oligomerization domain that extends into the female-specific part of DSX^F. The longest ORF of *Md-dsx*F1 starts at an AUG in position 62 in exon 2 and stops 95 bp downstream of the acceptor site of exon 4, coding for a protein of 397 aa (Fig. 2). The predicted protein contains a conserved OD1 domain with only five non-conservative changes in a stretch of 63 residues (Fig. 2a). This high degree of similarity extends to the amino-terminal end of the protein upstream of OD1. Likewise, the region corresponding to OD2 is very similar in sequence. Here, only seven non-conservative changes are found in a stretch of 64 residues. In particular, the female-specific domain (32 aa) encoded by exon 4 is virtually identical in both species (Fig. 2b). On the other hand, the region that links OD1 and OD2 is poorly conserved and, like in *Drosophila*, of low complexity containing an unusually large number of histidine, glycine and alanine residues (Fig. 2a).

The ORF of the male transcript *Md-dsx*M1 extends from the same AUG in exon 2 to a translational stop in exon 5 which is located 335 bp downstream of the acceptor site. The predicted protein encoded by *Md-dsx*M1 is 527 aa. In contrast to the female-specific

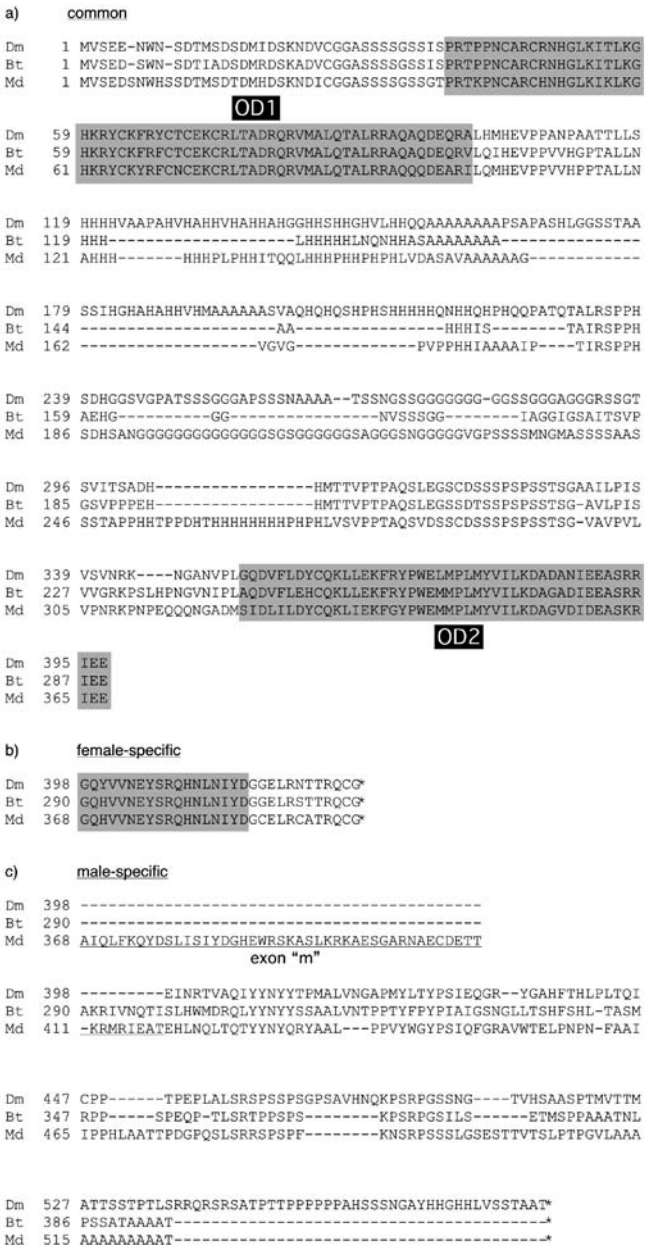

Fig. 2a–c Protein sequence alignment of *dsx* in *Drosophila melanogaster* (*Dm*; Burtis and Baker 1989), *Bactrocera tryoni*, (*Bt*; Shearman and Frommer 1998) and *Musca domestica* (*Md*). The sequence is divided into a part that is common to males and females (**a**), a female-specific part (**b**) and a male-specific part (**c**). The DNA binding domain (*OD1*) is *boxed in grey* in the aminoterminal region of **a**. Likewise the oligomerization domain (*OD2*) is *marked in grey* in the carboxyterminal of **a** and extends into the female-specific part (**b**). The sequence encoded by exon m is *underlined* in the male-specific part (**c**)

domain, the male-specific exon 5 is remarkably poorly conserved, displaying only very short stretches of similarity. In *Musca*, the male-specific part of the transcript starts with exon m, introducing an additional 49 aa upstream of exon 5 (underlined in Fig. 2c). This exon appears to be unique to *Musca*, since this sequence

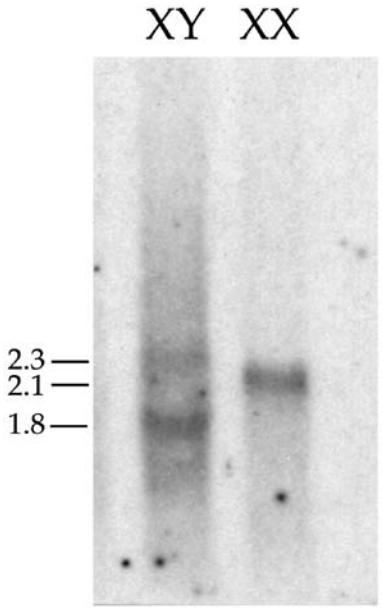

Fig. 3 *dsx* transcripts in adult male (*XY*) and female (*XX*) houseflies. Each lane contains 10 µg poly(A)$^+$ RNA prepared from adult flies and the blot was probed with the fragment *dsx*C (see Materials and methods)

Table 1 Presence of *Md-dsx* splice variants in different genotypes [+ abundant; *(+)* variable, low abundance; – not detected]

	*Md-dsx*M1 (2-3-m-5)	*Md-dsx*F1 (2-3-4)	*Md-dsx*C1 (2-3-m-4)
♀ Adults *(X/X)*	–	+	(+)
♂ Adults *(M^Y/X)*	+	(+)	+
♀ Adults *(M^{III}/M^{III}; F^D/+*	+	+	–
♂ Adults *(M^{III}/M^{III})*	+	–	–
♀ Adults *(F^{man}/F+)*	–	+	(+)
♂ Adults *(F^{man}/F^{man})*	+	(+)	–

is neither present in the male-specific domains of *dsx* in *Megaselia scalaris* (Kuhn et al. 2000) and *Bactrocera tryoni* (Shearman and Frommer 1998) nor in the lepidopteran species *Bombyx mori* (Ohbayashi et al. 2001).

Md-dsx is sex-specifically processed

Northern blot analysis of poly (A$^+$) RNA preparations verified the presence of transcripts of different sizes in male and female RNA samples when probed with an *Md-dsx* sequence common to both sexes (Fig. 3). In adult females, *dsx* produces a prominent transcript of about 2.1 kb, while in males two transcripts can be detected, a prominent band at 1.8 kb and a weak band at 2.3 kb. In addition, we performed a set of RT-PCR reactions with RNA prepared from males and females (listed in Table 1). By and large, we found that products amplified from females were predominantly composed of exons 2-3-4 and are thus likely to correspond to the major 2.1 kb

transcript. Amplification products from males were largely composed of exons 2-3-m-5-6. We therefore assumed this splicing variant to represent the predominant 1.8 kb transcript in males. In addition, our RT-PCR analysis revealed a less abundant splice variant composed of exons 2-3-m-4 which was found in male and female tissues (Table 1). As this variant, due to the incorporation of exon m, is 147 bp larger than the major female splice variant, it may correspond to the weak 2.3 kb transcript in males (Fig. 3). Sequence analysis of the main two splice variants indicates that pre-mRNA of *Musca dsx* is processed in a mode similar to that of *dsx* in *Drosophila*. However, it differs in one important aspect: the donor site of the common exon 3 is joined either to the female-specific exon 4 or to a male-specific exon m located upstream rather than downstream of exon 4 (Fig. 1b). We never detected any amplification products in which exon 3 is directly joined to exon 5 as seen in *Drosophila*.

Given that *Md-dsx* is regulated at the post-transcriptional level in response to absence or presence of *M*, the next question we addressed is whether this regulation is mediated by the switch gene *F*, the proposed principal target of *M* (Dubendorfer and Hediger 1998). To test this, we examined the structure of *Md-dsx* transcripts in strains that either carry the recessive loss-of-function allele of *F*, F^{man}, or the dominant gain-of-function allele of *F*, F^{D}. Houseflies homozygous for F^{man} develop into males even when *M* is absent (Schmidt et al. 1997). These no-*M* males produce only the male type of *Md-dsx* transcripts (Table 1). Evidently, absence of *F* activity results in the male mode of processing. Houseflies of the genotype *M/M;* F^{D}/+ develop into females even in the presence of *M*, because the dominant gain-of-function F^{D} allele overcomes the repression by *M* (McDonald et al. 1978). In these females, we detected *Md-dsx* transcripts of the female 2-3-4 composition (Table 1). Again, it is the state of activity of *F* that determines the splicing mode of *Md-dsx,* irrespective of whether *M* is present or absent. We therefore conclude that *Md-dsx* occupies a position downstream of *F* in the pathway.

The possibility that *Md-dsx* corresponds to *F* seems unlikely for the following two reasons. First, recessive loss-of-function alleles of *F* cause male development, whereas a dominant gain-of-function allele imposes female development. Accordingly, *F* is expected to be active in females but not in males (Dubendorfer et al. 2002; Hilfiker-Kleiner et al. 1993). *Md-dsx*, however, is active in males and females expressing sex-specific functions (see later). Second, genomic sequences around the regulated splice sites of *Md-dsx* in animals carrying either the gain-of-function F^{D} allele or the F^{man} allele did not differ from the corresponding wild-type sequences.

Functional study of *Md-dsx* in *Drosophila*

In *Drosophila*, DSX proteins act as transcriptional regulators that control the activity of genes responsible for the differentiation of sexually dimorphic traits. The sex-specific protein variants DSX^{F} and DSX^{M} behave antagonistically in the regulation of these target genes. For instance, DSX^{M} represses basal transcription of the yolk protein genes, whereas DSX^{F} stimulates transcription by binding to the same upstream promoter sequences (An et al. 1996; Coschigano and Wensink 1993; Erdman et al. 1996). It has been previously demonstrated that ectopic expression of DSX^{F} in XY males counteracts the activity of endogenous DSX^{M} and can impose some attributes of female differentiation (Baker et al. 1989; Waterbury et al. 1999). For instance, XY males carrying a constitutively active *dsxF* transgene contain substantial levels of vitellogenin in the hemolymph and produce a female profile of pheromones (Waterbury et al. 1999). To test whether the gene products of *Md-dsx* are capable of invoking the same responses, we introduced a transgene with *Md-dsx*F1 sequences driven by UAS into *Drosophila* XY males by P-element mediated transposition. When combined with an inducible hsp70-Gal4 driver, these males did not show any sign of sex reversal at the morphological level even when exposed to multiple heat pulses during their development. But, when these males were examined for the presence of vitellogenin, most lines tested gave a clear positive result (Fig. 4a). This demonstrates that *Md-dsx*F1 can overrule repression of yolk protein genes by endogenous DSX^{M} in *Drosophila*. The YP pattern in males though differs not only in levels but also appears less complex than that usually observed in females (Fig. 4a). A simple explanation for this could be differences in the tissue-specific expression of *yp* genes. The strong YP bands observed in female extracts largely derive from egg contents. The YPs in our transgenic males, on the other hand, are exclusively produced by the fat body. It is thus possible that the fat body in these males does not express the full repertoire of YPs.

In a reciprocal experiment, we tested for sex reversing effects by expressing the male variant *Md-dsx*M1 in *Drosophila* XX females. A UAS-*Md-dsx*M1 construct was introduced into XX females carrying the hsp70-Gal4 driver. These animals were exposed to multiple heat shocks during development and were examined as adult flies for the presence of sex transformed structures. Neither the anal nor genital regions were affected nor did this treatment result in the formation of male-specific bristles, the sex comb, on the forelegs. However, some lines displayed variable degrees of male-like pigmentation in the 5th and 6th tergite. The extent of pigmentation was most pronounced in lines carrying *Md-dsx*M1 under the control of an actin5C promoter (Fig. 4b). These lines were derived from a Hermes-based transposition, which is applicable in *Drosophila* (Guimond et al. 2003). Similar results were obtained in previous studies when *Drosophila* DSX^{M} was ectopically expressed using the actin5C promoter (Baker et al. 1989). Pigmentation in the posterior part of the female abdomen is normally repressed by DSX^{F} (Kopp et al. 2000). The presence of $MdDSX^{M1}$ thus appears to be capable of counteracting this repression allowing some level of pigments to be

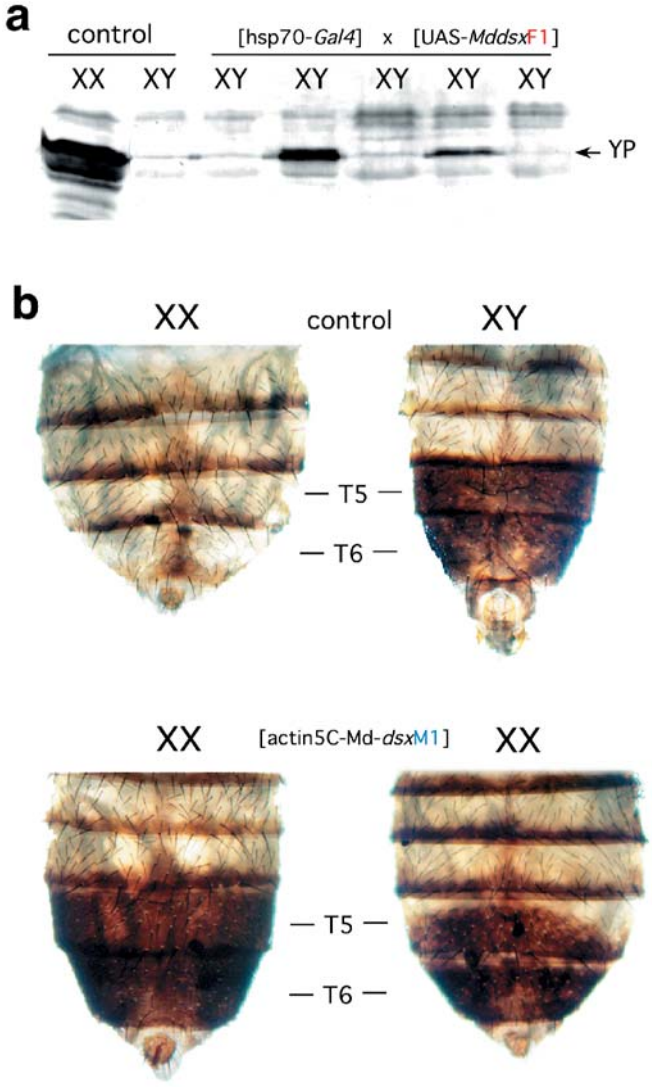

Fig. 4a, b *Md-dsx* expression in *Drosophila*. **a** Effects of *Md-dsx*F under the control of the hsp70 *Drosophila* promoter. Transgenic lines carrying a UAS-*Md-dsx*F1 construct were crossed to hsp70-Gal4 driver lines. Adult flies containing both constructs were exposed to several pulses of heat (1 h at 37°C) before hemolymph was removed for western blot analysis. Hemolymph samples collected from ten individuals were probed with a polyclonal antibody against *Drosophila* vitellogenin. The expected size of yolk polypeptides (*YP*) is indicated by an *arrow*. Controls are non-transgenic flies of the same *white*^{f118} strain. **b** Effects of *Md-dsx*M under the control of the actin5C *Drosophila* promoter. Preparations of abdominal tissues from non-transgenic males and females display the characteristic dimorphic pigmentation patterns in the most posterior tergites, T5 and T6. In transgenic XX individuals male pigmentation can be observed to a variable extent (XX; actin5C-*Md-dsx*M1)

produced. Taken together, these experiments show that forced expression of sex-specific protein variants of *Md-dsx* can elicit sex reverting effects in *Drosophila* suggesting that this gene has a similar function in regulating sexual differentiation in the housefly.

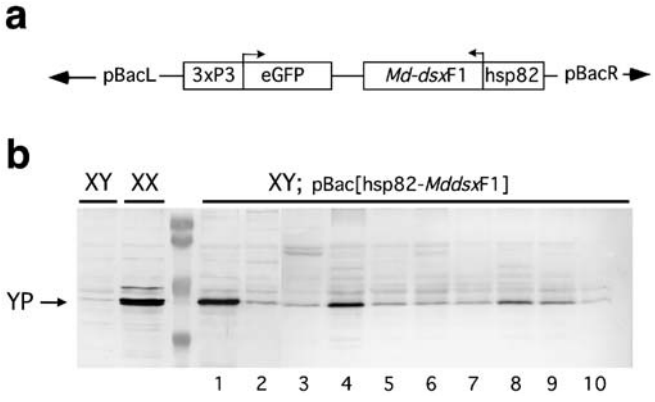

Fig. 5a, b Expression of vitellogenin in *Musca* males carrying the *Md-dsx*F1 transgene. **a** Schematic drawing of the piggyBac construct that was introduced into a *white* strain of *Musca*. The marker EGFP is driven by the 3xP3 promoter described in Horn and Wimmer (2000). *Md-dsx*F1 cDNA has been placed under the control of the promoter of the hsp82 gene from *Drosophila pseudoobscura*. *pBacL* and *pBAcR* indicate the locations of the inverted repeats of the *piggyBac* vector. **b** Western blot analysis of hemolymph from houseflies exposed to several rounds of heat shock (1 h at 42°C). In each lane the hemolymph protein content of an individual fly was electrophoretically separated and probed with a polyclonal antibody against *Musca* vitellogenin

Functional study of *Md-dsx* in the housefly

We generated an expression construct of the female-specific *Md-dsx*F1 cDNA under the control of promoter sequences of the hsp82 gene (Fig. 5a). This cassette was introduced into pBac{3xP3-eGFP}, a vector that was successfully applied to generate transgenic housefly lines in a previous study (Hediger et al. 2001). One line containing a stably integrated copy of this construct was recovered and tested for the presence of female traits in XY males after heat treatment. There was no visible evidence of sexual transformation in the external morphology even after multiple heat shock, but 10% of the transgenic males showed substantial levels of yolk protein in the hemolymph (Fig. 5b). We thus conclude that the *Musca* yolk protein genes are targets of *Md-dsx*. Whether this regulation is direct or indirect remains to be examined. In support of a direct transcriptional control is the presence of sites in the upstream regions of the *Musca* vitellogenin genes which have been shown to bind *Drosophila* DSX protein in vitro (Tortiglione and Bownes 1997).

Loss of *dsx* function in *Drosophila* prevents normal differentiation of dimorphic tissues, and instead leads to the formation of intersexual structures with no clear assignment to either the male or female fate (Baker and Belote 1983). It is expected that disruption of *Md-dsx* causes similar phenotypes in the housefly, if this gene indeed operates in the same pathway. We employed the RNAi technique to disrupt the activity of *Md-dsx*. This method of gene silencing has been successfully applied in *Musca* before (McGregor et al. 2001). dsRNA was prepared from the common region, from the female-

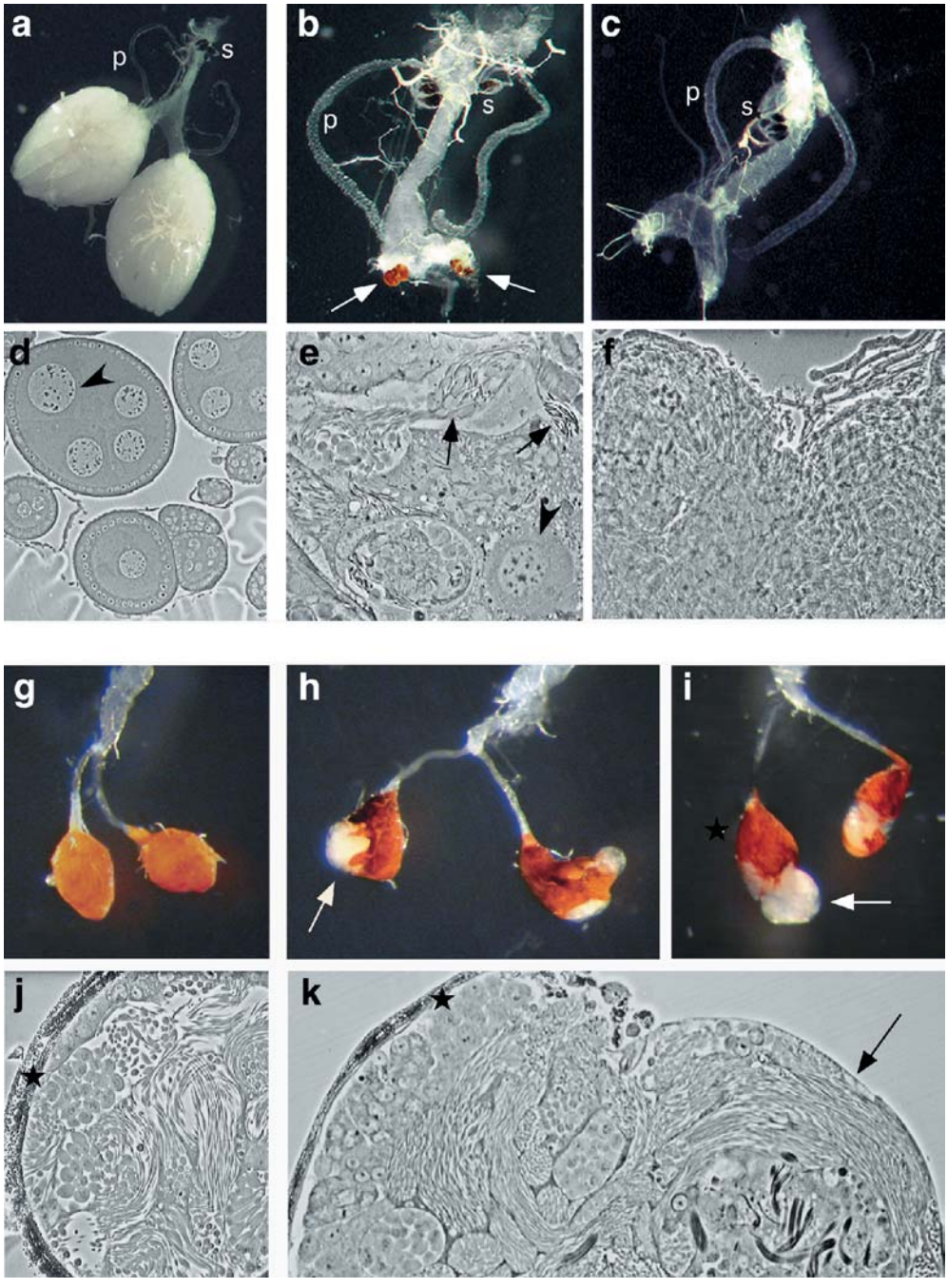

Fig. 6a–k Downregulation of *dsx* by RNAi in early embryogenesis affects gonadal differentiation. **a–f** Ovaries of *M/M; F^D/+* females. **a** A pair of normally developed ovaries of an untreated female (*p* parovarial glands, *s* spermathecae). **b** Markedly underdeveloped ovaries of a dsRNA-treated female. The *arrows* point to gonadal tissue with testis-like pigmentation. **c** In some cases gonadal tissue was completely absent at the tips of the oviducts. **d** Section through normally developed egg chambers. *Arrowhead* points to a polytene nurse cell nucleus. **e** Section through underdeveloped ovaries which are partially surrounded by testis-like tissue. *Arrows* indicate the presence of bundles of differentiating spermatids beneath this tissue. **f** Section through the non-developed ovary shown in **c**. **g–i** Testes of *M/M; +/+* males. **g** Normally developed testes of an untreated male. **h**, **i** Testes removed from males treated with dsRNA of *Md-dsx*. Note the presence of non-pigmented outgrowths at apical and lateral sites of the testis (*arrows*). **j** Section through a normal testis (*star* indicates epithelial tissue). **k** Section through a testis with apical outgrowth (*arrow*)

specific domain of *Md-dsx*F1 and from the male-specific domain of *Md-dsx*M1. Samples of these dsRNA were each injected into either the anterior or the posterior pole of syncytial blastoderm embryos of an autosomal M^{III} strain with marked chromosomes to distinguish genotypically male and female animals (see Materials and methods). About 12% of injected embryos survived to adulthood. A normal 1:1 ratio of males and females was obtained in all of the injected pools. Among these we did not observe any conspicuous abnormalities in the external morphology. Even injections of a concentrated mixture of all three dsRNA samples did not evoke visible phenotypes in adult flies of this strain. We next injected these dsRNA samples into embryos of the *M/M; F^D/+* strain. Again, surviving adults appeared normal in external morphology, but a substantial fraction (27%) of these *M/M; F^D/+* females contained one or two small underdeveloped ovaries with pigmented testis-like tissues located in the apical region (Fig. 6b). In some cases (8%), no gonads were formed at all (Fig. 6c). We examined the cytology of these abnormal ovaries in sections. In many cases, spermatid-like structures were found next to polytene nurse cells, suggesting that the germline content is a mixture of male and female differentiating cells (Fig. 6e).

Table 2 Effects of *dsx* RNAi in *Musca*. The total number of adult flies examined is in parentheses (*yp* yolk protein)

Strain	dsRNA template	♂		♀
		Testes with outgrowth	Yp production	Abberant ovaries
M^{III}/+	dsxC	0% (20)	0% (20)	0% (9)
	dsxF	0% (60)	0% (43)	9% (53)
	dsxM	7% (30)	0% (28)	0% (26)
M^{III}/+; pB{hsp82-dsxF}	dsxC	25% (20)	10% (20)	42% (38)
	dsxF	0% (27)	0% (26)	18% (34)
	dsxM	0% (19)	17% (52)	0% (5)

This intersexual phenotype is specifically caused by *Md-dsx* RNAi, since it was not observed in non-treated females of this strain or in females injected with dsRNA unrelated to *Md-dsx*. Not only the gonads of *M/M;* F^D/+ females appeared to be particularly sensitive to *Md-dsx* RNAi. Also, *M/M;* +/+ males of this strain show abnormal gonadal development after injecting dsRNA. In 47% of the cases we observed non-pigmented outgrowths at the apical ends of testes (Fig. 6h and i). Though sections through these abnormal testes did not reveal the presence of germ cells adopting a female fate (Fig. 6k), spermatid differentiation was clearly compromised, and males were sterile.

To test for sex-specific effects we injected dsRNA prepared from either only female (exon 4) or only male (exons m-5) templates into embryos of the autosomal M^{III} strain. Upon injection of *dsx*F dsRNA, 9% of +/+ females displayed abnormal ovarian differentiation, while testes of M^{III}/+ males were not affected and male fertility was close to normal (Table 2). On the other hand, injections of dsRNA with male-specific *Md-dsx* sequences disrupted testis development in 7% of examined M^{III}/+ males, while ovarian differentiation appeared normal in all cases (Table 2). We noticed that the number of flies with abnormal ovarian differentiation substantially increased when injecting a host strain that, in addition, carried a *Md-dsx*F1 construct driven by hsp82. Now, 42% of the females were affected when injected with dsRNA of the common part of *dsx* and 18% when injected with a dsRNA preparation of the female-specific part (Table 2). Again, injecting dsRNA of male-specific sequences had no visible effect in these females. These results give clear evidence for sex-specific requirements for *Md-dsx* in gonadal development. Together with the data of ectopic expression of *Md-dsx*, they indicate that *Md-dsx* plays an essential role in controlling sexual differentiation of the housefly.

Cis-elements required for TRA/TRA2 mediated activation of the female splice site are present in *Musca dsx*

In *Drosophila*, the female-specific processing of *dsx* depends on the activities of the upstream regulators *tra* and *tra2* (Hedley and Maniatis 1991; Hertel et al. 1996; Ryner and Baker 1991; Tian and Maniatis 1993). The products of these genes bind to *dsx* pre-mRNA to direct the utilization of the splice acceptor site of the female-specific exon 4. This acceptor site is preceded by a polypyrimidine stretch interrupted by several purines and, therefore, considered to be suboptimal for recruiting components of the spliceosome (Hedley and Maniatis 1991; Ryner and Baker 1991). Binding of TRA/TRA2 protein complexes to six 13 nt repeats (dsxRE) in the 3′ untranslated region of exon 4 enables this acceptor site to be recognised and utilized by the generic splicing machinery. In *Md-dsx*, the female-specific splice site is located at the very same position and the 3′ UTR of *Md-dsx* exon 4 contains a cluster of three sequences with substantial similarity to the dsxRE of *Drosophila* (Table 3). Another structural requirement for TRA/TRA2 binding, namely the purine-rich enhancer element (PRE) close to the dsxRE, is also present in the 3′UTR of *Md-dsx* exon 4 (Fig. 7). The dsxREs and the PRE are clustered in a region starting 500 bp downstream of the acceptor site of exon 4 and flanked on both sides by poly(A) signal sequences (arrows in Fig. 7). Amplification of female *Musca* cDNAs with a sense primer in exon 4 and oligo(dT) yielded different fragments that correspond to the sizes expected when poly(A) signals upstream and downstream of the dsxRE/PRE cluster are utilized. This specific arrangement of splice enhancer elements downstream of the regulated splice site suggests that female exon selection depends on an activation mechanism similar to that in *Drosophila*. The polypyrimidine sequence upstream of the activated female splice site, however, neither significantly deviates from the Y_nNYAG consensus nor does it appear suboptimal when compared to the polypyrimidine tract preceding the acceptor site of the male exon 5 (Table 4).

Table 3 Comparison of dsxRE present in the female-specific exon of *Musca dsx*

Drosophila

T C T T C A A T C A A C A
T C T A C A A T C A A C A
T C A T C A A T C A A C A
T C A A C G A T C A A C A

Musca

A C A A C A A T C A A C A
T C A T C A A T C A A C A
T C A A C A A C C A A C A

Table 4 Comparison of splice acceptor sites. Shown are the sequences of the splice acceptor sites upstream of the male-specific exons m and 5 and of the female-specific exon 4 of *Musca domestica* and *Drosophila melanogaster*. Purines in the polypyrimidine tract are *boxed in grey*

```
M. dom. "m" ♂  ----------------ccccctaaacaauuuucuu a cag CCAUACA
M. dom. "5" ♂  ----------------cuuuaucgacuuaccuuca a uag CUACAGA
D. mel. "5" ♂                              uucuguuaucc c cag CUCGAGU

M. dom. "4" ♀  --(582 bp)-------ucucuucuucaauucuguu u uag GACAACA
D. mel. "4" ♀  --(114 bp)-------ucucucgaucugaucuaaa c cag GCCAAUA

CONSENSUS                          YYYYYYYYYYYYYYYYY N CAG
```

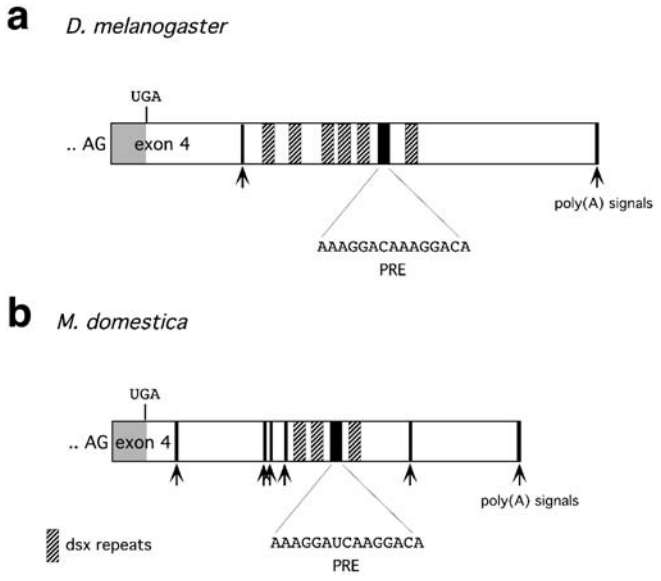

Fig. 7 Distribution of *dsx* repeats in the female-specific exons of *Drosophila* (**a**) and *Musca* (**b**). Translational stops (*UGA*) and potential poly(A) sites are indicated. Location of the 13 nt *dsx* repeats are marked as *hatched boxes*, and the purine-rich sequence (*PRE*) as a *filled box* in the 3′UTR

Another significant deviation from the *Drosophila* situation is the existence and differential splicing of an additional exon m upstream of the female-specific exon 4. This sequence is only included in the two male splice variants, which argues against a simple mechanism in which female-specific splicing of *Md-dsx* is based only on activation of the female acceptor site. The polypyrimidine tract preceding exon m is interrupted by several purines and deviates more significantly from the Y_nNYAG consensus than the polypyrimidine tract of exon 4 (Table 4). No potential TRA/TRA2 binding sites have been found in the vicinity of this exon.

Discussion

We have isolated and characterized a gene in the housefly *M. domestica* with structural and functional homology to the sex-determining gene *dsx* in *D. melanogaster*. The functional correspondence was demonstrated by misexpression studies in *Drosophila* and *Musca*. The female-specific variant, $MdDSX^F$, induces yolk protein synthesis, a typical female physiological response, when expressed in *Drosophila* and *Musca* males. The male variant $MdDSX^M$ causes male-like pigmentation of the posterior tergites in *Drosophila* females. Apart from these subtle sex-reversing effects, the transgenic flies remained morphologically normal. The failure of these transgenes to induce complete sex reversal can be attributed to the antagonistic activity of endogenous *dsx*. A previous report (Waterbury et al. 1999) demonstrated that the final sexual phenotype depends on the relative amount of DSX^M and DSX^F expressed in *Drosophila* cells. For instance, XY individuals expressing DSX^F from a transgene can be gradually transformed into pseudofemales when the dose of endogenous *dsx*, which expresses DSX^M, is decreased. Hence, the female-promoting activity of $MdDSX^F$ in transgenic *Musca* males may be antagonized by the two copies of intact endogenous *Md-dsx* that produce $MdDSX^M$. This explains why these transgenic males are fertile and do not display any detectable female traits apart from the presence of yolk in the hemolymph. It appears that, like in *Drosophila*, genes expressing physiological traits are more responsive to changes in the relative amounts of DSX^M and DSX^F than genes which express morphological traits (Baker et al. 1989; Waterbury et al. 1999). The same observation was made in the lepidopteran species *Bombyx mori* (Suzuki et al. 2003). The female-specific activity of the *dsx* homologue, *Bmdsx*, elicits a physiological female response, namely synthesis of vitellogenins and hexameric storage proteins, and downregulation of pheromone-binding proteins that are preferentially expressed in males, but does not result in a morphologically visible female transformation in *Bombyx* males (Suzuki et al. 2003).

Regulation of yolk protein genes in *Musca*

In standard *Musca* strains, the three yolk protein genes (*Mdyp1*, *Mdyp2*, *Mdyp3*) are transcriptionally repressed in males (White and Bownes 1997). It is thus conceivable that ectopic $MdDSX^F$ in males relieves this repression by a direct interaction with the promoter of these genes. In *Drosophila*, transcriptional regulation of yolk protein genes is the best characterized function of *dsx*. Both variants, DSX^F and DSX^M, can bind to sequences in the promoter region of the yolk protein genes, *yp1* and *yp2*

(Burtis et al. 1991; Coschigano and Wensink 1993). A zinc finger-like DNA-binding domain in the common part of the DSX polypeptides is responsible for binding to several sites in the fat body enhancer (FBE; Erdman and Burtis 1993). Though both proteins bind to the same enhancer, they elicit opposite responses: DSX^F binding results in activation, and DSX^M binding in repression of transcription (An and Wensink 1995a, 1995b). This difference in molecular behaviour is defined by the second oligomerization domain (OD2) which interacts with distinct sets of cofactors (An et al. 1996). Our results suggest that *Musca* DSX proteins have a conserved function in *yp* gene regulation. First, both *Musca* variants share a DNA binding domain (OD1) that is almost identical in amino acid sequence to that of *Drosophila*. Second, in *Drosophila*, $MdDSX^F$ can relieve repression of *yp* transcription imposed by endogenous DSX^M. This is most likely achieved by effective competition for the same binding sites in the FBE. To activate *yp* transcription, $MdDSX^F$ must be capable of interacting with cofactors in *Drosophila* that normally bind to DSX^F. The high degree of sequence conservation found in OD2 of $MdDSX^F$ and DSX^F suggests that this oligomerization domain is responsible for these specific interactions.

A direct role of *Md-dsx* in regulating transcription of the *Musca yp* genes is supported by the presence of potential *dsx* binding sites in the promoter regions of *Mdyp1* and *Mdyp3* (Tortiglione and Bownes 1997; C. Siegenthaler, unpublished results). Furthermore, some of the sites in *Mdyp1* were able to bind *Drosophila* DSX in gel-mobility shift assays (Tortiglione and Bownes 1997). Given the high structural similarity of the DNA binding domain, it seems likely that these sites can also interact with MdDSX. None-the-less, the finding that the promoter sequences of *Mdyp1* cannot confer sex-specific expression in *Drosophila* questioned the contribution of *Md-dsx* in controlling yolk protein synthesis (Tortiglione and Bownes 1997). The authors proposed that, different from *Drosophila* where *dsx* is the primary determinant, *Musca* involves the endocrine system and uses ecdysteroids as a key regulator for sex-specific expression of YP. This mode of hormonal control allows the cyclical laying of eggs to be synchronized with cyclical synthesis of YP. Accordingly, females exhibit a distinct peak of ecdysteroid concentration during egg cycles, whereas the level in males remains continuously low (Agui et al. 1985). The strong correlation between levels of ecdysteroids and YP suggested that *Md-dsx* plays no or only a marginal role in the control of YP expression. However, we demonstrated that expression of $MdDSX^F$ is sufficient to elicit YP synthesis even in *Musca* males where ecdysteroid levels remained low (C. Siegenthaler, unpublished results). We therefore propose that *Md-dsx* is an integral component and interacts with ecdysteroids in this regulation. Binding of MdDSX may influence the response threshold of *yp* genes to ecdysteroids, e.g. binding of $MdDSX^F$ may lower the threshold to a level where even the low ecdysteroid concentration in males is sufficient to trigger expression of the *yp* genes.

Role of *Md-dsx* in gonadal differentiation

Homologues of *dsx* are found in a growing number of insect species (Kuhn et al. 2000; Ohbayashi et al. 2001; Pane et al. 2002; Shearman and Frommer 1998). In all reported cases, it has been shown that the *dsx* homologue produces sex-specific mRNA variants suggesting that *dsx* is widely used as a double switch in the sex determination pathway. Our RNAi study provides evidence that *Md-dsx* is indeed involved in important aspects of dimorphic development. Interference of *Md-dsx* in embryos causes abnormal differentiation of gonads in males and females. This result did not come as a surprise as correct assignment of gonadal soma to become either testes or ovaries is likely to be one of the first implementations of the sex-determining program. Hence, loss of *dsx* at an early stage is expected to disrupt normal gonadal differentiation and to cause ambiguity with regard to the sexual fate of this tissue. The occurrence of pigmented testicular-like tissue in gonads of RNAi-treated females can thus be explained as a direct result of this ambiguous state. Likewise, the unpigmented outgrowths found in the gonads of RNAi-treated males may have derived from cells with ambiguous or unspecified assignment. Intersexual and undifferentiated tissues are typical hallmarks of the phenotypes described in *dsx* mutant *Drosophila* flies (Nothiger et al. 1987). The mutation also affects the internal reproductive system in *Drosophila* females (Hildreth 1964). Often small degenerated ovaries were found in XX; *dsx*/*drx* animals similar to those that we observed in *dsx* RNAi-treated *Musca* females. In line with a sex-specific activity of *Md-dsx*, the specific repression of male or female messages led to abnormal gonadal differentiation only in the affected sex. We infer from this that *Md-dsx* is essential for the correct sexual development of embryonic gonads.

Injections of *Md-dsx* dsRNA in embryos left the external morphology of the developing adults unaffected. This does not necessarily mean that *Md-dsx* does not control the sexual differentiation of the imaginal cells. Rather, the injected material may not persist into later developmental stages, and therefore may not interfere with intrinsic *dsx* RNA. Genetic studies in *Drosophila* have unambiguously demonstrated that *dsx* does also control the sexual differentiation of imaginal disc cells in advanced developmental stages (Baker and Ridge 1980; Belote and Baker 1982).

Regulation of *Md-dsx*

The production of sex-specific transcripts in *Drosophila* is achieved by differential splicing. In males, exon 4 is skipped by default, and instead the downstream exons 5 and 6 are included in the mature transcript. In females, the presence of the splice regulatory activities of TRA/TRA2 promotes the incorporation of exon 4 in the mature transcript. This pattern of sex-specific splicing is also observed in the Queensland fruitfly *Bactrocera tryoni* and

the Phorid fly *Megaselia scalaris* where it occurs in equivalent positions of the corresponding *dsx* genes (Kuhn et al. 2000; Shearman and Frommer 1998). Moreover, Pane et al. (2002) demonstrated in a recent report that female-specific splicing of the *dsx* gene in the Mediterranean fruit fly *Ceratitis capitata* depends on the activity of the *tra* homologue. The presence of putative TRA/TRA2 binding sites in the female-specific exon of *dsx* in *Bactrocera* and *Megaselia* gives further support to the notion that female exon selection by activation is common in dipteran insects. In the lepidopteran species *Bombyx mori*, the *dsx* homologue is subjected to the same pattern of sex-specific processing, but the underlying mechanism appears to be different (Suzuki et al. 2001). Here, female splicing represents the default mode when tested in HeLa nuclear extracts, and also the female exon is devoid of putative TRA/TRA2 binding sites. The authors therefore proposed that the female exon is selectively repressed in male silk moths by a yet unknown mechanism (Suzuki et al. 2001).

The situation in *Musca* is more consistent with the activation mode, primarily because putative splice enhancing sequences are present in the female exon. However, the existence of another differentially spliced exon upstream of the female exon adds a level of complexity not observed previously. Its absence in female transcripts suggests that an additional level of control exists which selectively prevents the recognition of exon m in female cells. It is feasible that activation of the female splice acceptor and repression of exon m are mediated by the same mechanism. For instance, sex-specific processing of the *tra* homologue in *Ceratitis* involves 5′ and 3′ splice site selection and exon skipping (Pane et al. 2002). The clustering of several TRA/TRA2 binding sites in the vicinity of these regulated splice sites suggests that *Ceratitis* TRA has an autocatalytic function which is capable not only of activating splice sites but also of repressing splice sites in the *tra* pre-mRNA. Furthermore, the autoregulatory activity of TRA2 in spermatogenesis of *Drosophila* mediates repression of a specific splice site rather than its activation (Chandler et al. 2003; Mattox and Baker 1991). On the other hand, it is also possible that differential processing of exon m is uncoupled from that of the female exon. Instead, it may be omitted by default but become specifically activated in male cells. In particular, the poor match of its 3′ splice site to the polypyrimidine consensus lends some support to this idea. In this scenario, correct processing of *Md-dsx* may thus not only rely on the use of female-specific splice activators but also on male-specific activators.

Evolution of sex determination pathways

The existence of different sex-determining mechanisms in natural populations of *M. domestica* makes the housefly a particularly suited system for studying evolutionary changes in sex determination pathways (Dubendorfer et al. 1992). We believe that these variations reflect minor changes in an otherwise well conserved pathway. By identifying the genes in the *Musca* pathway, we aim at an understanding of the principles of the underlying genetic control and, by comparison with sex-determining genes in other species, of how such pathways evolve. Thus far, our results are consistent with the model proposed by Wilkins (1995) that sex determination pathways evolve from bottom to top in a retrograde fashion. *dsx* appears to be a common terminal regulator in all hitherto analysed pathways. But this extent of congruence seems to halt at the level of the upstream regulator *F*. While, in *Musca*, this gene seems to be the direct target of the primary signal, *Drosophila* recruited yet another upstream switch, *Sxl*. The reason for this added level in the cascade is not known. A sex-determining function of *Sxl* has so far only been demonstrated in members of the genus *Drosophila*, and its recruitment to the pathway is thus believed to be a rather recent event (Schutt and Nothiger 2000). This level of understanding may prove helpful for the identification of corresponding sex switches in other insects, in particular in species of medical and agricultural relevance. This work will contribute not only to an understanding of the evolutionary forces that shape sex-determining pathways, but also to the design and application of new genetic tools for a use in population control programs of pest insects.

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